"Evaluation of the Hypoxia Inducible Factor-1α-β Protein Heterodimerisation Inhibition Using Low Molecular Weight Compounds as Lead Molecules"

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Cancer is the deadliest disease in the world, which stands second after cardiovascular disease. About 23% of people die annually in USA due to cancer. In India, it accounts for about 7% deaths. There will be 15.0 million new case of cancer by 2020 as population increases up to 7. 5million. In India prevalence of cancer is around 2.5 million, 8, 00,000 new cases and 5, 50,000 deaths were estimated per annum. In Karnataka, there are roughly 1.5 lakh cancer patients at any given moment, with about 35,000 new cancer cases added each year(1). The internal factors (Genetic mutations, hormonal, poor immune conditions) and external factor (food habits, industrialization, over growth of population, lifestyle-related) are major causes for such a high rate of cancer incidence. In addition to this, ignorance, delayed diagnosis and lack of adequate medical facilities contribute to increased death due to cancer. Most of the cancer treatment strategies are failed due to aggressiveness and metabolic reprogramming in solid as well as haematopoietic cell carcinomas. The metabolic reprogramming is the major cause of chemo and radiation therapy resistance in cancer cells(Rob A. Cairns *et al.* 2011). Hypoxia is the major phenotype in extensive grown solid tissue and haematopoietic cell carcinomas, where metabolic reprogramming is common(2). Hypoxia is observed in cells, due to extensive growth of tissue and cells located apart from vasculature in haematopoietic carcinoma. The cells located 150μm experience hypoxia. The tumour tissues adapted to hypoxic condition by the influence of hypoxia inducible factors (HIF). HIFs induce several counter genes that help in tumour adaption to hypoxic stress. HIFs are the family of bHLH-PAS family of transcription factors, which are hetero dimers of alpha and beta subunits. The beta subunits are well known as aryl nuclear hydrocarbon receptor translocator (ARNT) that is not affected by oxygen level(3). The alpha subunits are unstable and undergo ubiquitin mediated proteosomal degradation

during low oxygen atmosphere. There three isoforms of alpha subunits namely, 1, 2, 3 and coded by different genes.

Aim and Objectives of the Study

Protein-Protein interactions in hypoxia are the best drug target in the current scenario. We aim, to screen potential inhibitors of HIF-1α-β heterodimerisation and its validation. So, the objectives of this studies are,

a) To understand the molecular mechanism behind HIF's interactions with other proteins in the hypoxia signaling pathway.

b) Effect of selected low molecular weight compounds on HIF-1 dimerization and transactivation.

Summary of the work done:

Hypoxia-inducible factor -1α is an important regulator of metabolic reprogramming and chemo- and radiation-therapy resistance. HIF-1α binds to HRE in DNA and transcribes genes involved in metastasis, the Warburg effect, pH homeostasis, angiogenesis, cell death, and differentiation. The current investigation found that LMCs with drug-like characteristics were able to successfully inhibit HIF-1α and interaction. LMCs also suppress other components of the hypoxia signalling pathway, which aids in HIF-1α stability (HSP90AA1) and radiation resistance (HSP90AA1) (TIGAR). Thesis is divided into four chapters.

Chapter 1: Introduction

The first chapter provides a general review of the research that has been done so far, as well as the gaps in earlier studies, therefore adding to the existing knowledge. The first chapter of the thesis also provides insight about cancer treatment strategies and their limitations. In this introduction we also summarised about recent advances in Low

molecular weight compounds as anti-cancer agents and targeting protein -protein interaction in cancer.

Chapter 2: Literature Review

The evaluation of literature begins with hypoxia and cancer connections, then moves on to structural detail of HIF-1 and other HIFs. This section deals about role of HIF-1α and β in cancer, protein-protein interaction hypoxia system. This also summarizes about studies reported about inhibitors of Protein-Protein interaction in hypoxic system.

Chapter 3: Materials and Methods

The third chapter contains information on the materials and experimental procedures employed in this study. This chapter explains the methods of *in-silico* studies such as sequence analysis, molecular docking and simulation. *In-vitro* studies includes cytotoxicity, immunoblotting, Co-immunoprecipitation (co-IP) and Immunofluorescence.

Chapter 4: Results and Discussions

The findings were presented using tables, graphs, and figures. The results of all the experiments reported in relation to the hypothesis are covered in this chapter.

Sequence analysis of Hypoxia inducible factors

This study is an attempt to appreciate the evolutionary changes of protein HIF-1α and the detailed sequence variations across the set of species considered. Key substitutions in the oxygen-dependent hydroxylation of the ODD and the consequence of the same towards its recognition to VHL enable us to explain its criticality with respect to HIF-1α half-life and its activity. Comparisons of the HIF-1α sequence among cancer-resistant animals, such as BMR and NMR, and cancer-prone animals, such as human, mouse, and rat, suggest derivation of probable clues towards potential risk factors for

cancer. Except DNA binding- bHLH domain other domains showed less conservation. The sequence analysis also provided supporting evidence to develop inhibitor. The amino acids that interact determine the selectivity of the molecules.

Structural basis of HIF-1α and HSP90AA1 interaction

The interaction of HIF-1α with HSP90, prevent HIF-1α from hypoxic stress induced degradation(4,5). There is no evidence of structural basis of HIF-1α and HSP90 interaction. Here we showed the structural details of HIF-1α and HSP90 interaction. The GLU269 involved in the formation of salt bridge with ARG260 of HSP90. There are 18 hydrogen bond and 24 non–bonded interaction found in this interaction. GLU245, TYR254, ARG258, GLN299, GLN304, GLN320, THR322, TYR325, THR327, GLN333, CYS334, VAL336, VAL338, TYR340 these are residues of HIF-1α involved in interaction with β subunit. Katschinski et al, 2004 said, same PAS-B involved in interaction with HSP90. But, till date no one reported residual information about HSP90.

Destabilizing the HIF-1α by targeting HSP90 using small molecules

HSP90AA1 chaperone molecule palys key role in stabilization and folding. It is key chaperone in hypoxic stabilization of HIF factor that involves in tumour tissue aggressiveness and treatment failure. So, Targeting HSP90 will be the promising strategy to knockout the tumour adaption. We found, screened natural compounds having better binding affinity than reported drug and also pass the criteria of Lead like molecules. Pharmacophore Model generated is useful in de-novo designing of new lead molecules.

Screening of natural products as inhibitor of TIGAR

HIF-1α induces the translocation of novel protein TIGAR (TP53 (tumour protein 53)-induced glycolysis and apoptosis regulator) cytoplasm to inner mitochondrial

membrane. Where, TIGAR induces the activation of Hexokinase 2(HK2). HK2 maintains the ADP level in the membrane, there by inhibits the apoptosis. In this study, we screened natural product that bind to TIGAR with high affinity. We found 24 lead molecules which is having docking score less than -7. MMGBSA also supports the docking results.

***In-silico* screening for Inhibitors of HIF-1 Dimerization**

HIF-1α and HIF-1β interaction is key step in hypoxia induced downstream gene expressions. We screened Cambridge anti-cancer compound library and natural products for developing new scaffold that interfere the HIF-1α-β interaction. From entire study set we selected two lead compounds (D1 and D2) for *in-vitro* studies.

Assessment of cytoxicity of selected lead

Compound D1 and D2 were tested for cytoxicity against HEK293, MCF7, PC3 and DU145 cell lines.

Table 1. IC50 Values of D1 and D2 against HEK293, MCF7, PC3 and DU145

Cell line	IC50 value	
	Compound D1	Compound D2
DU145	12 µmol	7 µmol
PC3	8 µmol	5 µmol
MCF	15 µmol	13µmol

Disruption of Dimerization of HIF-1α with HIF-1β and its inhibition

Co-Immunoprecipitation (co-IP) assays confirmed that compound D1 and D2 inhibits the dimerization of HIF-1α with HIF-1β. As *in-silico* studies depict, the compound D1 and D2 were successful in inhibits in heterodimerisation in *in-vitro* conditions also. DU145 cells were exposed to hypoxia condition prior to drug treatment. After, 24hours of drug treatment cells were lysed and proteins were pulled using HIF-1α antibody

tagged A/G coupled agarose beads. Tagged protein eluted and performed western blotting. IP with anti-HIF1α showed the absence of interaction with HIF-1β. Same membrane was subjected to stripping and performed WB using anti-1β.

Inhibition of HIF-1α and its degradation

Immunoblotting of whole cell lysate revealed that HIF-1α in cells undergone degradation. Under induced hypoxia, D1 and D2 reduce the protein level in dose dependent manner. This may be due to the interruption of PAS-B domain and HSP90. The HSP90 is key Chaperone protein that stabilizes the HIF-1α in hypoxia.

We are proposing a novel way of targeting Hypoxia inducible factor 1 α in caner. PAS domain is an important domain that shares surface area with its subunit, HIF-1 beta as well as with chaperone protein HSP90. Placing nitro-heterocyclic compound at PAS domain inhibits the transactivity of HIF1 α and pushes it into degradation. This way, compound D1 and D2 became promising lead molecule that can be used in treatment solid tumors.

In summary, we concluded that targeting HIF-1α is a better way of developing anti-cancer drug. The stability and dimerisation of HIF-1α with HSP90 and HIF-1β is key step in HIF-1α transactivity. In hypoxic stress, HIF-1α stabilized by HSP90, Inhibiting of this interaction results in degradation of HIF-1α. The compounds that binds to C-terminal ATP binding pocket of HSP90, disrupt the HIF-1α – HSP90 interaction. Our, compounds were shown binding affinity towards C-terminal ATP binding site. These natural compounds may reduce the interaction of HIF-1α – HSP90 interaction. PAS-B domain of HIF-1α involved in interaction with HSP90. Same PAS-B of HIF-1α interacts with HIF-1β to form active heterodimeric complex. In next strategy we screened compound that can

binds to the PAS-B domain of HIF-1α and inhibits the heterodimerisation. In *in-silico* studies and Co-immune-precipitation of HIF-1α and β showed inhibition of HIF-1 heterodimerisation. Also, D1 and D2 inhibit the level of HIF-1α protein in prostate cancer cell lines.

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List of Publications

- Poyya, J., Kumar, D. J., Nagendra, H. G., Dinesh, B., Aditya Rao, S. J., and Joshi, C. G. (2021). Receptor based virtual screening of potential novel inhibitors of tigar [TP53 (tumour protein 53)-induced glycolysis and apoptosis regulator. Medical Hypotheses, 110683. doi:10.1016/j.mehy.2021.110683.

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Paper Presented in Conference

- Poyya, J., Dinesh, B., & Joshi, C. G., (2021) *In-Silico* screening of small molecules as inhibitor of NRF2. Presented at International conference on "Recent trends and advances in biomedical sciences - an integrated approach" held during on 25-27 November 2021 organized by Nitte University and Indian Association of Biomedical Scientists (IABMS).

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Publications Co-Authored

- Dinesh B, Monisha N, Shalini H, Pratap GK, Poyya J, Jayanth H, et al. Antibacterial Activity of Silver Nanoparticles Synthesized Using Endophytic Fungus-*Penicillium cinnamopurpureum*. Spectroscopy Letters 2021. https://doi.org/10.1080/00387010.2021.2010764.

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